Exhibit E4

Diversity of MICA (PERB11.1) and HLA haplotypes in Northeastern Thais

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Abstract: MICA or PERB11.1 is a polymorphic major histocompatibility complex (MHC) class I-related gene located 46 kb centromeric of the HLA-B gene in the HLA class I region. It is expressed mainly in gut epithelial cells, keratinocytes, endothelial cells, fibroblasts and monocytes, and is upregulated by heat stress. MICA has been found to interact with γδ T cells, αβ $CD8^+$ and natural killer (NK) cells bearing the NKG2D/DAP10 receptor. The MICA gene displays a high degree of polymorphism with at least 54 alleles. In the present study, polymorphic exons 2, 3 and 4 of the MICA gene were analyzed using sequencing based typing (SBT) in 255 unrelated healthy northeastern Thais. Thirteen previously reported MICA alleles were detected. MICA*008, *010, *002 and *019 were highly predominant with the allele frequencies of 21.4%, 18.2%, 17.6% and 15.3%, respectively. Five of these 13 MICA alleles show significantly different frequencies from those of the Japanese and Caucasian populations. Interestingly, MICA052, which is a very rare allele in other populations, was prevalent with the allele frequency of 8.2%, mainly on the HLA haplotype carrying HLA-B*13 in this population. Strong linkage disequilibria were observed between MICA and HLA-B, as similarly observed in other populations, namely MICA*010-B*4601, MICA052-B*13, MICA*002-B*5801, and MICA*019-B*15 (1502, 1508, 1511, 1515, 1528, 1530). A large variety of three-locus (MICA – HLA-B – HLA-Cw) and six-locus (HLA-DQB1 – HLA-DRB1 – MICA – HLA-B – HLA-Cw – HLA-A) haplotypes were recognized in the northeastern Thai population. This is the first report on MICA allelic distribution in Southeast Asian populations. These data will provide the important basis for future analyses on the potential role of the MICA gene in disease susceptibility and transplantation matching in Southeast Asian populations.

The telomeric region of the human major histocompatibility complex (MHC) includes the MHC class I chain-related (MIC) (1) or PERB11 (2) gene family consisting of seven members: MICA (PERB11.1), MICB (PERB11.2), MICC (PERB11.3), MICD (PERB11.4), MICE (PERB11.5), MICF and MICG (3, 4). Of these, only MICA and MICB are expressed, encoding cell surface glycoproteins of 383 amino acids. The MICA gene is located approximately 46 kb centromeric of HLA-B. MICA molecules are composed of three extracellular domains (α1, α2 and α3), a transmembrane segment and a carboxy-terminal cytoplasmic

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tail similar to other HLA class I molecules. However, MICA shares only an average of 21, 19 and 34% amino acid identity in the α1, α2 and α3 extracellular domains, respectively, with classical HLA class I proteins. Further, MICA and HLA class I gene products differ regarding tissue distribution, regulation of expression, association with β_2-microglobulin and immunological function.

MICA has a diverse tissue distribution. It is transcribed in fibroblast, epithelial cell lines, gastrointestinal epithelium, freshly isolated keratinocytes, endothelial cells and monocytes but not in B lymphocyte (5, 6). MICA is also expressed in some lung, breast, kidney, ovary, prostate and colon carcinoma (7). Expression of the gene is not affected by γ-interferon stimulation but is regulated by promoter heat-shock elements and strongly induced upon cell stress, thus MICA may function as an indicator of cell stress (8, 9). MICA is recognized by a subset of γδT cells that predominate in intestinal and other epithelial sites (8, 9), which suggests that MICA facilitates γδT-cell detection of infected, damaged, or otherwise stressed epithelial cells, thus forming part of the innate immune system (10). Recently, MICA has been reported to be a ligand for an activating receptor, NKG2D, which is expressed on most natural killer (NK) cells, CD8+αβT cells and γδT cells (11). This interaction might activate NK-cell and T-cell responses against MICA-bearing tumors.

The MICA gene displays a high degree of genetic polymorphism in exons 2, 3 and 4, amounting to at least 54 alleles (12–14). In contrast to classical class I genes, the polymorphic sites are distributed throughout the domains but outside the peptide-binding groove of the MHC class I molecules (12). They are defined by a total of 40 nucleotide substitutions, 30 of which are nonsynonymous; 5 of 8 in exon 2, 15 of 16 in exon 3 and 10 of 16 in exon 4 (13). Genetic polymorphism was also recognized in the transmembrane region including indels and variable number of the GCT triplet repeats (4). However, information on the MICA diversity in human ethnics is limited, being derived mainly from the Caucasian and Japanese populations. In the present study, we have used sequencing based typing (SBT) to define MICA alleles in the native Northeastern Thai (NET) population. The results were compared with those from the Caucasian and Japanese populations. Linkage disequilibria among MICA and HLA alleles were also investigated to identify representative HLA haplotypes in the NET population.

Material and methods

Genomic DNA

Peripheral blood cells were collected from 255 unrelated healthy Northeastern Thai individuals. All of them were interviewed for their ancestors. Their families are living in the northeast of Thailand for at least two generations. Genomic DNAs were extracted from buffy coat by the salting-out method (15). Their HLA class I (HLA-A, -B and -C) and class II alleles (HLA-DRB1 and -DQB1) were typed by the polymerase chain reaction-sequence-specific primer (PCR-SSP) technique (Romphruk et al., in preparation). Genomic DNA from an HLA homozygous tissue culture cell line (HTLC), BM15 with MICA*004 was employed as a standard DNA in the SBT protocol.

PCR amplification and sequence determination of the MICA gene

A 2.2-kb MICA gene fragment from exon 2 to exon 5 was amplified by a pair of PCR primers, 5MICA and 3MICA (12). PCR conditions using these primers were described previously (16) with some modifications. Namely, PCR was carried out in a 50-μl reaction using 100–200 ng of DNA and 0.5 units AmpliTaq™ DNA polymerase in the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 95°C for 15 min, followed by 35 cycles of 95°C 30 s, 61°C for 1 min, 72°C for 2 min and final elongation at 72°C for 10 min.

Cycle sequencing

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and subjected to sequence determination using the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS (Applied Biosystems). Each of exons 2, 3 and 4 was separately sequenced on both DNA strands using six sequencing primers as described by Katsuyama et al. (16). The products were purified by Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ, USA). The reactions were run on the ABI 377 or ABI 310 Sequencing System (Applied Biosystems) and the results were analyzed using the ABI Sequence Analysis and Navigator Softwares. Raw sequencing data were manually inspected for confirmation and alleles were assigned by alignment with known 54 MICA alleles of the extracellular domains (13, 14).

Statistical analysis

Allele frequency (AF) was calculated using the following formula: %AF=sum of each individual allele/2N ×100. Haplotype frequency (HF) and linkage disequilibrium (D) were calculated by the maximum likelihood method assuming the Hardy-Weinberg law. This value indicates an association between the two loci. Significance of two-locus association was assessed by the Chi-square test. The

significance of the distribution of alleles between NET and other populations was tested by *Pc* value (corrected *P*-value) after the Chi-square method.

Results

MICA allele frequencies in Northeastern Thais

The distribution of MICA alleles was determined by sequencing based typing in 255 unrelated healthy Northeastern Thai individuals (NET). Thirteen alleles were recognized in this population. The allele frequencies (AF) of the MICA gene are listed in Table 1. The common alleles were MICA*008 (21.4%), MICA*010 (18.2%), MICA*002 (17.6%) and MICA*019 (15.3%). In contrast, MICA*020 (0.4%) and *016 (0.2%) were infrequent. Most of the "blank" allele cases revealing the frequency of 7.8% possibly represent a MICA homozygote, but some or a significant fraction of them may correspond to a MICA deletion allele linked to HLA-B*4801 in Asian populations (17, 18). In fact, B*4801 was not so frequent, but was recognized with the allele frequency of around 0.5% in NET (Romphruk et al., unpublished data).

Comparison of MICA allele frequencies among NET, Japanese and Caucasians

The allelic distribution of the MICA gene in NET was compared with those of the Japanese (16) and Caucasian (19) populations (Table 2). As a result, it must be most notable that MICA052 (MICA052 has not be officially recognized and so * is not attached between MICA and 052) (14) was considerably prevalent with the frequency of 8.2% in NET, but absent in Japanese or Caucasians. Further, the significantly high frequencies of MICA*010 (the second most frequent allele in NET), MICA*018 and MICA*019 were found in NET as compared to the Japanese and Caucasian populations by the corrected *P*-value test. On the other hand, the frequencies of MICA*008 were significantly decreased in NET as compared to those of both populations, however, MICA*008 was the most frequent allele amongst all the three populations including NET. In addition, the significantly low frequency of MICA*004 and MICA*009 was observed in NET, only when compared to the Japanese population. There was no MICA*001, MICA*007 or MICA051 (MICA051 has not be officially recognized) (14) allele in NET, which was present with the low frequency in the Japanese and Caucasian populations.

Allele frequencies of MICA in 255 Northeastern Thais (NET)

MICA	*n*	% AF
002	90	17.6
004	18	3.5
008	109	21.4
009	12	2.4
010	93	18.2
012	16	3.1
016	1	0.2
017	11	2.2
018	31	6.1
019	78	15.3
020	2	0.4
026	7	1.4
052	42	8.2

Table 1

Comparison of MICA alleles of NET with those of Japanese and Caucasians

	%AF		
MICA	NET *n*=255	Japanese (16)** *n*=130	Caucasians (19)** *n*=242
001	0.0	0.0	3.0*
002	17.6	14.6	13.0
004	3.5	9.2*	6.0
007	0.0	1.2	2.0*
008	21.4	30.8*	55.0*
009	2.4	16.5*	3.0
010	18.2	10.8*	5.0*
011	0.0	0.0	2.0
012	3.1	12.3	2.0
016	0.2	0.0	2.0
017	2.2	0.0	4.0
018	6.1	0.0*	2.0*
019	15.3	3.5*	0.0*
020	0.4	0.0	1.0
026	1.4	0.0	0.0
051	0.0	0.0	2.0*
052	8.2	0.0*	0.0*

* significantly different (*Pc*<0.05)
** reference no.

Table 2

Linkage disequilibrium and haplotypes between MICA and HLA-B in NET

Linkage disequilibrium parameters (delta values) and haplotype frequencies (HF) between MICA and HLA-B alleles were calculated in NET. Strong associations were observed between these two loci, as previously reported in the Japanese population (16). MICA – HLA-B associations with statistical significance at the *P* level of less than

Significant MICA and HLA-B associations in 255 NET

MICA	HLA-B	*n*	% HF	Delta	Chi-square
002	35	20	3.9	2.3	21.5
002	38	14	2.8	1.7	17.5
002	5801	49	9.6	7.1	94.8
004	44	17	3.3	3.1	224.8
008	27	26	5.1	3.3	27.5
008	4001	33	6.5	4.6	45.5
008	07 (02–07)	13	2.5	1.1	4.7
008	39/6701	21	4.1	2.3	14.4
008	40 (02,04–06)	16	3.1	1.8	16.9
009	51/5201	10	2.0	1.9	99.3
010	4601	75	14.7	11.6	160.6
010	15a	18	3.5	2.4	17.0
012	5401	4	0.8	0.8	60.7
012	55/56	11	2.2	2.0	99.0
017	57	11	2.2	2.1	232.8
018	18	30	5.9	5.1	158.3
019	1521	8	1.6	1.2	16.3
019	15a	13	2.5	1.4	6.9
019	15b	46	9.0	6.5	87.5
026	07 (02–07)	6	1.2	0.9	52.0
052	13	41	8.0	7.6	215.4

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

Table 3

0.05 corresponding to a delta-value of equal to or more than 0.8 are listed in Table 3 with 21 representative haplotypes. The most common haplotype was MICA*010-B*4601 (14.7%), followed by MICA*002-B*5801 (9.6%), MICA*019-B*15(02,08,11,15,28,30) (9.0%) and MICA052-B*13 (8.0%). The NET population was characterized by a large variety of associations of MICA with HLA-B, such as MICA*002, MICA*008, MICA*010, MICA*012 and MICA*019 showing linkage disequilibria with multiple HLA-B alleles as shown in Table 3.

Multi-locus haplotypes in NET

Frequencies of three-locus (MICA – HLA-B – HLA-Cw) and six-locus (HLA-DQB1 – HLA-DRB1 – MICA – HLA-B – HLA-Cw – HLA-A) haplotypes were calculated by incorporating the above MICA – HLAB haplotypes into the known HLA class I and class II haplotypes in NET, and those with more than 0.4% (more than two individuals carrying the same haplotype) are listed in Tables 4 and 5, respectively. They revealed the presence of diversed and con-

Possible MICA – B – Cw haplotypes in 255 NET

MICA	HLA-B	HLA-Cw	*n*	%HF
008	07 (02–07)	07 (01–03)	11	2.2
026	07 (02–07)	15 (02,03,05)	5	1.0
052	13	0304	22	4.3
052	13	04	18	3.5
008	13	0602	5	1.0
019	15a	04	9	1.8
010	15a	07 (01–03)	6	1.2
019	15b	04	4	0.8
019	15b	08	36	7.1
010	15b	07 (01–03)	2	0.4
019	1521	04	7	1.4
008	18	07 (01–03)	4	0.8
019	18	07 (01–03)	5	1.0
018	18	0704	28	5.5
018	18	04	2	0.4
008	27	0304	17	3.3
008	27	07 (01–03)	2	0.4
019	27	1202	3	0.6
002	35	04	13	2.6
002	38	07 (01–03)	10	2.0
002	39/6701	07 (01–03)	2	0.4
008	39/6701	07 (01–03)	18	3.5
008	40 (02,04–06)	15 (02,03,05)	5	1.0
008	40 (02,04–06)	0304	2	0.4
008	4001	0303	3	0.6
008	4001	0304	7	1.4
008	4001	04	6	1.2
008	4001	07 (01–03)	14	2.8
004	44	07 (01–03)	16	3.1
010	4601	01	73	14.3
020	5001	0602	2	0.4
019	51/5201	07 (01–03)	2	0.4
002	51/5201	14	6	1.2
009	51/5201	14	5	1.0
009	51/5201	15 (02,03,05)	3	0.6
012	5401	01	4	0.8
012	55/56	01	6	1.2
012	55/56	1202	2	0.4
002	55/56	1203	2	0.4
017	57	0602	11	2.2
002	5801	0302	42	8.2

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

Table 4

Possible MICA and 5- locus HLA haplotypes in 255 NET *Table 5*

DQB1	DRB1	MICA	B	Cw	A	*n*	%HF
0501	1001	026	07 (02–07)	15 (02,03,05)	29	4	0.8
0501	1502	052	13	04	11	4	0.8
0502	1602	052	13	0304	11	5	1.0
02	07	008	13	0602	30	5	1.0
0502	1502	010	15a	07 (01–03)	34/66	4	0.8
0301	1202	019	15b	08	11	10	2.0
0601	1501	019	15b	08	24	6	1.2
0601	1502	019	1521	04	34/66	6	1.2
0501	1502	019	18	07 (01–03)	0203	3	0.6
0501	1502	018	18	0704	0203	5	1.0
0501	1502	018	18	0704	24	8	1.6
0301	1202	018	18	0704	24	5	1.0
0301	1101	008	27	0304	24	2	0.4
0301	1202	008	27	0304	24	3	0.6
0501	1502	008	27	0304	24	6	1.2
0501	1502	002	38	07(01–03)	11	2	0.4
0501	1502	002	38	07 (01–03)	24	2	0.4
0302	0405	008	39/6701	07 (01–03)	0203	5	1.0
02	07	004	44	07 (01–03)	33	13	2.5
0303	09	010	4601	01	11	6	1.2
0502	1202	010	4601	01	11	6	1.2
0303	09	010	4601	01	24	3	0.6
0303	09	010	4601	01	0207	21	4.1
0502	1202	010	4601	01	0207	10	2.0
0502	1401	010	4601	01	0207	4	0.8
0502	1602	010	4601	01	0207	2	0.4
0303	07	017	57	0602	01	4	0.8
02	0301	002	5801	0302	33	26	5.1
06 (04–07)	1302	002	5801	0302	33	3	0.6
02	0301	002	5801	0302	11	4	0.8

15a=15 (01, 04–07, 12, 14, 19, 20, 24, 25, 26N, 27, 32–35)
15b=15 (02, 08, 11, 15, 28, 30)

served MICA- HLA-B – HLA-Cw haplotypes. The three most common three-locus haplotypes were MICA*010 – B*4601 – Cw*01 (HF=14.3%), MICA*02 -B*5801 – Cw*0302(HF=8.2%) and MICA*019 – B*15(02,08,11,15,28,30) – Cw*08 (HF=7.1%). The two most common six-locus haplotypes were DQB1*02 – DRB1*0301 - MICA*002 – B*5801 – Cw*0302 – A*33 (5.1%) and DQB1*0303 – DRB1*09 -MICA*010 – B*4601 – Cw*01 – A*0207 (4.1%). Further, other than those haplotypes, as many as 38 distinct three-locus and 28 six-locus haplotypes were recognized in more than two NET individuals, indicating generation of a large variety of allelic combinations among the HLA loci in NET.

Discussion

The MICA gene shows an extensive degree of genetic polymorphism, which is slightly more concentrated in the α2 domain, resembling classical HLA class I molecules. However, in contrast to classical class I molecules MICA polymorphism is equally diverse within the α3 domain. Further, sequence variation at each polymorphic position in the MICA gene has been limited to biallelic substitution in contrast to many multi-allelic ones in the classical MHC class I genes. The structure of MICA molecules revealed by X-ray crystallography confirms

the general configuration of the molecules with an MHC class I fold (20, 21). However, the MICA polymorphic residues are positioned on the outer edge of an antigen-binding cleft, apparently bordering an invariant ligand-binding site, unlike MHC class I molecules. Therefore, actual role of polymorphism in MICA molecules has yet to be determined.

In order to investigate genetic polymorphism in the extracellular domains of the MICA gene in the NET population, SBT was adopted in this study. Consequently, MICA*008 was the most common allele, but its allele frequency showed a significant decrease as compared with those of the Japanese and Caucasian populations (see Table 2). The MICA*008 allele is highly dominating in Caucasians (AF=55%), being associated with B*0801, B*0702, B*4402 and B*4001 (19). In Caucasian, its predominant abundance and representation on multiple haplotypes could indicate that MICA*008 is the ancestral allele with subsequent recombinations and point mutations producing further allelic diversity (19). In the NET population, MICA*008 (AF= 21.4%) is mostly associated with unique HLA-B alleles (B*27 and B*39/6701) different from that in the Caucasian population. MICA*008 in the NET population carries the A5.1 allele in the TM (transmembrane) domain (unpublished data), as similarly observed in other populations (16, 22), which possibly produces a truncated MICA protein because of a frame-shift mutation due to a one-base insertion in exon 5.

MICA*010 and *019, which are different from MICA*008 by double- and single-nucleotide substitutions, are also prevalent with AFs of 18.2% and 15.3% in NET, respectively. These two MICA alleles might have been generated from MICA*008 and then conserved in the Thai population. However, they were associated with TM (A5) and HLA-B alleles (B*4601 and B*15) different from MICA*008 in this population. Additionally, MICA*010 carries a nucleotide substitution at position 17 in exon 2, which results in a proline instead of an arginine by substitution at amino acid position 6 in the α1 domain. This substitution has been recently established to abolish cell surface expression of the MICA*010 allele by blocking a β-sheet hydrogen bond with the histidine carbonyl group at position 27 on the β2 strand. Moreover, this substitution is incompatible with β-sheet secondary structure, thus likely interfering with protein folding (20, 23). Thus far, we have recognized six individuals homozygous for the MICA*010 – B*4601 – Cw*01 haplotypes, and all of them were apparently healthy. This might be consistent with the fact that no MICA/MICB knock-out individuals homozygous for the MICA deletion – MICB null (MICB0107N) haplotype manifested any clinical symptoms (17, 24, 25).

Interestingly, MICA052, which is identical to MICA*007 except for a single-nucleotide substitution at nucleotide position 751 (G to C), was detected only in the NET population, but not in the Japanese or Caucasian population (see Table 2). MICA*007 was not present in NET. The MICA052 sequence was submitted to GenBank as Accession No AH007476, but its ethnic origin is unknown. This allele was in a strong linkage disequilibrium with B*13 (41/42) in NET (see Table 3). HLA-B*13, which is quite common in NET, is known to be linked to three Cw alleles as major haplotypes (B*13-Cw*0304, B*13-Cw*04 and B*13-Cw*0602). Among them, the former two-locus B*13-Cw*0304 and B*13-Cw*04 haplotypes were associated with MICA052 (see Table 4). Collectively, these facts imply that MICA052 is a relatively old allele introduced to or newly generated in the B*13-associated haplotype in NET before the diversification of the B*13-Cw haplotype. Further, the existence of a large number of the two-locus, three-locus and six-locus MICA associated haplotypes may also suggest the ancient origin of MICA alleles, possibly before recent creation of HLA diversity.

Natural killer cells have been shown to express MHC class I molecules recognizing receptors that are thought to function primarily as negative or positive signalling receptors in the course of the self-non-self discrimination. Among them, NKG2D is supposed to be the putative ligand as an activating receptor for MICA molecules. It remains to be determined whether NKG2 can distinguish MICA alleles and whether other NK receptors such as KIR2 (CD158a/b), a killer cell inhibitory receptor, can recognize two alternative epitopes on the HLA-Cw molecules (26). If so, MICA allelic distribution and/or linkage disequilibria between MICA and other class I alleles may give important information on donor-recipient matching and the outcome of transplantation. In conclusion, we carried out SBT of MICA in the NET population showing a large variety of the allelic and haplotypic distributions, which will provide the important basis for future analyses on the potential role of the MICA gene in disease susceptibility and transplantation matching in South East Asian populations.

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